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CELL BIOLOGY – IMMUNOLOGY – PATHOLOGY

Small heat shock protein alteration provides a mechanism to reduce mesangial cell contractility in diabetes and oxidative stress

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Alteration to small heat shock proteins provides a mechanism to reduce mesangial cell contractility.

Background. Small heat shock proteins are expressed in many tissues and are proposed to regulate actin filament dynamics when dissociated into small aggregates and phosphorylated in a p38 mitogen-activated protein kinase (p38MAPK)-dependent manner.

Methods. p38MAPK activity and small heat shock protein-25 (Hsp25) were determined in glomeruli from rats with experimental diabetes induced by streptozotocin administration and in isolated glomeruli exposed to a free radical stress. Contractile responsiveness of mesangial cells was determined by the serum-induced contraction of cell-embedded type I collagen gels.

Results. In experimental diabetes, there is an activation of p38MAPK, a decrease in the size of Hsp25 molecular aggregates, from large to small homo-oligomers, and an increase in the phosphorylation of Hsp25. In control glomeruli, a free radical stress, H_2O_2, activated p38MAPK and increased Hsp25 in a concentration-dependent manner. Additionally, H_2O_2 decreased the contractility of cultured mesangial cells concomitant with an increase in Hsp25 phosphorylation and a reduction in Hsp25 aggregate size. These effects were significantly reduced by SB202190, an imidazole-derivative cell-permeable inhibitor of p38MAPK.

Conclusions. It has been proposed that the generation of oxygen-derived free radicals in diabetes may be linked causally to a loss of glomerular contractile reactivity and thus hyperfiltration in the early stages of diabetes mellitus. This study provides a mechanism for alteration of mesangial cell contractile responsiveness through phosphorylation of Hsp25 and may be a mechanism underlying abnormalities in glomerular hemodynamics in diabetes and in the presence of free radical stress.

Several lines of evidence, including the activation of transcription factors, overexpression of growth factors, and activation of protein kinase cascades, suggest that early in the course of diabetes, multiple pathways may converge to increase reactive oxygen species and a diabetes-induced oxidative stress [1]. This may arise from the increased generation of free radicals that accompanies glucose autoxidation and the process of oxidative glycoxidation [2] or the condition of "pseudohypoxia," in which superoxide radicals are formed in the mitochondrial response to the altered reduced pyridine nucleotide ratios that follow glucose metabolism through the polyol pathway [3]. A major challenge in the investigation of the microvascular complications of diabetes has been to identify which of many hyperglycemia-driven biochemical changes contribute to microvascular dysfunction. Stress proteins/heat shock proteins are highly conserved, abundantly expressed proteins that act as molecular chaperones in many conditions of cellular stress, including heat shock [4]. An increase in one group, small heat shock proteins (small Hsp, murine/rodent 25 kd, human 27 kd), is proposed to confer tolerance to a number of stressors in addition to the stress of heat shock [5]. Under diabetic conditions, there is a loss of glomerular mesangial cell contractility with alteration in the actin cytoskeleton [6]. We have investigated conditions under which mesangial cell contractile response may be defective, and in this study, we describe a possible mechanism involving phosphorylation of small heat shock proteins. Heat shock proteins are expressed in many tissues, and increased expression of small Hsps enhances the survival of cells exposed to oxidant stress [7]. Protection may be conferred by two properties of the small Hsps. When present as large, unphosphorylated aggregates, Hsp27 controls intracellular reactive oxygen species and cellular glutathione (GSH) content in Hsp27-transfected cells [8]. In addition, when dissociated from large to small aggregates and phosphorylated in a p38 mitogen-activated protein kinase (p38MAPK)-dependent manner following exposure to stress, small Hsps are proposed to regulate actin filament dynamics and to stabilize mi-

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crofilaments [9–12]. It has been proposed that during stress, this serine phosphorylation-induced conformational change in small aggregates (homo-oligomers) of Hsp regulates the activity of this protein as an actin-binding protein, preventing actin depolymerization and providing stress fibers as an adaptive response to the stress condition [10, 13]. Small Hsp phosphorylation has been described in cardiac myocytes exposed to an oxidative stress [11]. In cardiac and skeletal muscle, small Hsps colocalize with the actin filaments of the sarcomere I-bands and M-lines [14], but a functional significance of this has not been established beyond the proposal that this may play a role in the assembly of the thin-filament array of the sarcomere and/or be involved in contraction [5]. In the presence of raised glucose or following diabetes, isolated mesangial cells and glomeruli show an impaired contractile response to a number of pressor agents, including arginine vasopressin [6], endothelin 1 [6, 15, 16], angiotensin II [15, 17], or bradykinin [18], or to KCl [19]. Activation of the MPK-activated protein kinase MAPKAPK-2, a target for the dual tyrosine/threonine-phosphorylated p38MAPK, is an ubiquitously described mechanism for small Hsp phosphorylation [9, 11, 20], although phosphorylation *in vitro* can be by protein kinase C (PKC), specifically the δ isoform [21]. Our preliminary work has shown activation of p38MAPK in the renal glomerulus in experimental diabetes [abstract; Dunlop M *Diabetes* 47(Suppl 1):A51, 1998]. We hypothesized that p38MAPK-related phosphorylation of small Hsps in response to glucose-induced reactive oxygen species could alter the stability of the actin cytoskeleton and, subsequently, mesangial cell contractility in diabetes. The aim of this study was to determine the level and aggregate size of heat shock protein-25 (Hsp25) in the glomerulus and in cultured mesangial cells and to relate this to glomerular and mesangial cell contractility.

METHODS

Induction of diabetes and preparation of glomeruli and cultured mesangial cells

Preparation of diabetic animals, isolation of glomeruli, and culture of mesangial cells from explanted glomeruli were performed as described previously [22, 23]. Briefly, diabetes was induced in male Sprague Dawley rats (weighing approximately 200 g) by a single intraperitoneal injection of streptozotocin (STZ). Diabetes was maintained for three weeks before the commencement of experiments. In each of four experimental cohorts, one group of diabetic animals received twice daily subcutaneous injections of Ultratard insulin to give a total of 25 IU/kg/day. These cohorts have been characterized previously [23]. Over four cohorts with nine animals in each treatment group, blood glucose levels were 6.0 ± 0.5 mmol/L (control), 17.7 ± 0.6 mmol/L (STZ diabetic), and 9.6 ± 0.7 mmol/L (STZ diabetic + insulin). From each control and treatment group, four animals were chosen at random for individual glomerular preparations. Body weights and kidney weight of these animals were documented (Table 1). Kidneys from the remaining animals in the control and the untreated STZ-diabetic groups were pooled to provide a bulk glomerular preparation.

Glomeruli were prepared from control, STZ diabetic, and insulin-treated diabetic rats by differential sieving of kidney cortex. Approximately 10,000 to 15,000 glomeruli were isolated from each rat kidney, with no significant differences between treatment groups. Glomeruli were used immediately or incubated in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal bovine serum (FBS; vol:vol) for the indicated additions for up to 18 hours. Mesangial cells were grown from explanted glomerular cores, prepared by the digestion of intact glomeruli from control rats with 0.5 mg/mL collagenase type V for 10 minutes at 37°C. Explanted glomerular cores were maintained in DMEM containing 20% FBS (vol:vol), and the resultant mesangial cells were used at passage 3. Mesangial cells were maintained in the previously mentioned media containing 0.5% FBS (vol:vol) for 18 hours prior to use. When present, buthionine sulfoximine (BSO), a γ-glutamyl cysteine synthetase inhibitor, was included over this time. Prior to use, cells were washed rapidly with two changes of warmed medium, and additions made for the indicated times before cells were washed with ice-cold phosphate-buffered saline (PBS) and lyzed in the buffers described in the next section of this article.

Immunoprecipitation and Western immunoblotting

For immunoprecipitation and Western immunoblotting, lysates were prepared from freshly isolated glomeruli or cultured mesangial cells in a buffer, pH 7.5, containing 150 mmol/L NaCl, 5 mmol/L Tris, 2 mmol/L egtazic acid (EGTA), 1 mmol/L sodium vanadate, 40 mmol/L sodium fluoride, 50 mmol/L β-glycerophosphate, 1% Triton X-100, 0.25% deoxycholate, 0.15 U/mL aprotinin, 10 μU/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Hsp25 was immunoprecipitated from lysates, equalized for protein content, by incubation overnight at 4°C with antibody SPA-801, a rabbit polyclonal antibody raised against recombinant mouse Hsp25, followed by a 45-minute incubation with protein A sepharose. After washing the immunoprecipitates in PBS containing 0.2% Triton X-100, immunoprecipitated proteins were removed from protein A sepharose by boiling and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% wt/vol acrylamide). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes in Towbin

Table 1. Body weight and kidney weight determinations

Treatment group	Control	Insulin-treated diabetic	Untreated diabetic
Body weight *g*	322.8 ± 3.1	334.0 ± 4.1	276.0 ± 5.6[a]
Kidney weight *g*	1.62 ± 0.03	1.67 ± 0.04	2.08 ± 0.05[a]

Body and kidney weights were measured at sacrifice. Results are expressed as mean ± SEM for 16 animals in each treatment group (4 animals in each treatment group over 4 separate cohorts).

[a] Significant difference from control and insulin-treated diabetic group

buffer (25 mmol/L Tris, pH 8.3, 192 mmol/L glycine, 20% methanol) in the absence of SDS, probed with SPA 801 antibody, and detected by enhanced chemiluminescence (ECL). For Western immunoblotting of phospho-p38MAPK and p38MAPK, proteins were separated by SDS-PAGE as described earlier in this article, transferred to PVDF membranes and probed sequentially with phospho-specific p38MAPK antibody (#9211S) and p38MAPK antibody (#9212) and detected by ECL. Phospho- and nonphospho-p38MAPK cell extracts (#9213) from C-6 glioma cells were included as controls.

Sizing of Hsp25 molecular aggregates

Lysates for gel filtration were prepared in a buffer, pH 7.4, containing 20 mmol/L NaCl, 20 mmol/L Tris, 5 mmol/L $MgCl_2$, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and 50 mmol/L β-glycerophosphate and separated for size on a 60 cm column of Sephacryl S300. The column being eluted with lysis buffer with Triton X-100 was omitted. Fractions from these columns were separated by SDS-PAGE (reducing conditions) followed by Western blotting (antibody SPA 801) and ECL detection.

Glomerular and mesangial cell [^{32}P] labeling of Hsp25

For glomerular ^{32}P labeling, glomeruli were maintained in DMEM containing 0.5% FBS with [^{32}P]orthophosphate (2.0 mCi/mL) for four hours. Mesangial cells were maintained in 100 mm dishes under the same low serum conditions with [^{32}P]orthophosphate (0.1 mCi/mL) for 24 hours. When present, BSO was included in the labeling period. After this time, glomeruli and mesangial cells were washed rapidly with three changes of warmed medium, and additions were made for the times indicated. Lysates were prepared for immunoprecipitation, SDS-PAGE, and transfer to PVDF membranes for autoradiography before immunoblotting by the methods described earlier in this article using antibody to Hsp25 (SPA-801).

Contraction of mesangial cell-embedded collagen gels

To determine gel contraction, mesangial cells (2.5×10^5 cells/mL) were suspended in a solution of DMEM and type I rat-tail collagen (3 mg/mL) and 0.5 mL was placed in 24-well tissue culture dishes, which had previously been coated with 0.2 mL of 0.67% agarose and allowed to gel for one hour at 37°C in an atmosphere of 5% CO_2 in air. Serum-free DMEM (0.5 mL) was placed over the gels at this time, and incubation was continued for 24 hours. Additions were made in this serum-free medium at 2× the final concentration. After 24 hours, the medium was removed and replaced with DMEM containing any previous additions and 3% FBS. The gel contraction that occurred over the subsequent six hours was monitored by inspection, and at six hours, the floating gel was removed intact to an acetate sheet for the determination of the planar surface area. This method is based on an extensively investigated procedure and documented previously by Ailenberg et al [24] and Zent, Ailenberg, and Silverman [25].

Contraction of isolated glomeruli

To determine glomerular contractile responsiveness, isolated glomeruli were maintained in DMEM containing 0.5% FBS for four hours at 37°C in an atmosphere of 5% CO_2 in air. When present, H_2O_2 and SB202190 were included over this time. Glomeruli were washed rapidly in three changes of warmed medium and placed in six-well culture plates and incubated in the basal state or with the addition of KCl (50 mmol/L for 10 min) or bradykinin (100 nmol/L for 10 min). Over this time, glomeruli were viewed with a Nikon Diaphot microscope with an attached photographic system (FX35DX, HFX-IIA), and nine fields were photographed over the last 90 seconds of incubation. Glomerular planar surface area was calculated from measurement of glomerular diameter, determined from an average value of four diameters per glomerulus, measured on projected slide images using a superimposed micron scale. All intact glomeruli within the nine photographic fields (50 to 65 glomeruli in each of two independent glomerular preparations) were measured for each condition.

Determination of protein oxidation

As a measure of free-radical oxidation, the carbonyl content of glomerular protein was determined. This well-described spectrophotometric assay measures protein-bound 2,4-dinotrophenylhydrazone formed by reaction of protein dicarbonyls with 2,4-dinitrophenyhydrazine (DNPH) [26, 27]. Briefly, lysates were prepared from control and diabetic glomeruli as described for West-

ern immunoblotting (mentioned previously) and were treated for 15 minutes with 1% streptomycin sulfate to remove contaminating nucleic acid. Lysates containing 500 to 600 μg protein were incubated with 0.2% DNPH in 2 N HCl for one hour at room temperature in the dark. Blank incubations were performed in the presence of 2 N HCl alone. Proteins (derivatized or blank) were precipitated by addition of trichloracetic acid (final concentration 10%) and centrifuged at 11,000 *g* for five minutes. The resulting pellet was washed three times by the addition of ethanol:ethyl acetate 1:1, vol:vol) with vortexing followed by centrifugation after each addition. The resulting washed protein pellet was resuspended in 6 mol/L guanidine-HCl, and the absorbence was measured at 370 nm. Carbonyl content was calculated as nmol carbonyl per mg protein using the molar absorptivity of carbonyl-reacted DNP-hydrozone, 22,000 $mol^{-1} \cdot cm^{-1}$.

Reagents

Ultratard insulin was from Novo Nordisk A/S (Copenhagen, Denmark). STZ, collagenase Type V, buthionine sulfoximine, β-glycerophosphate, leupeptin, phenylmethylsulfonyl fluoride, Sephacryl S-300-HR, streptomycin sulfate, and 2,4-dinitrophenylhydrazine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMEM was from Trace Biosciences Pty. Ltd. (Castle Hill, New South Wales, Australia). FBS was from Commonwealth Serum Laboratories (Parkville, Victoria, Australia). [^{32}P]orthophosphate was from Bresatec (Adelaide, South Australia). SB202190, [4-(4-Fluophenyl)-2-(4-hydroxyphenyl)-5-5(4-pyridyl)1H-imidazole] was obtained from Calbiochem-Novabiochem Pty. Ltd. (Alexandria, New South Wales, Australia). Anti-Hsp25 antibody (SPA-801) was from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Polyclonal phospho-specific p38MAPK antibody (#9211S), p38MAPK antibody (#9212), and phospho- and nonphospho-p38MAPK cell extracts (#9213) were from New England Biolabs, Inc. (Beverley, MA, USA). Chemiluminescence reagent was from NEN Life Sciences Products Inc. (Boston, MA, USA). Rat tail collagen was from Boehringer Mannheim GmbH (Mannheim, Germany). Bradykinin was from Auspep Pty. Ltd. (Parkville, Victoria, Australia).

Statistics

Single comparison analysis was by Student's unpaired *t*-test (two-tailed). Where multiple comparisons were made, significance was determined by analysis of variance in conjunction with Fisher's least-squares difference, and significance was assumed at $P < 0.05$.

RESULTS

Detection of p38MAPK in glomerular extracts

The level of p38MAPK protein and activated phospho-p38MAPK was determined by Western immu-

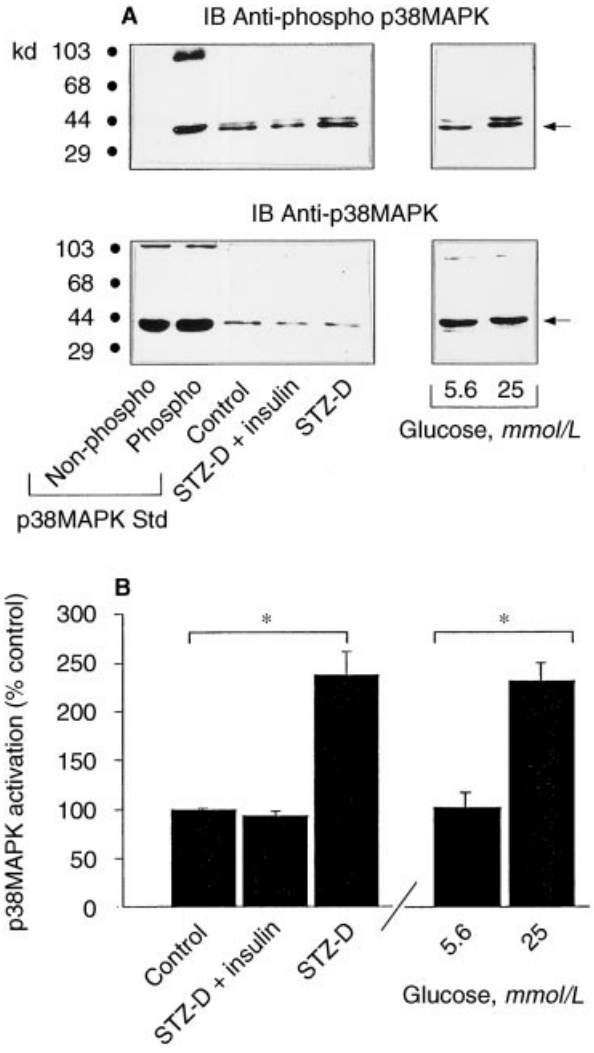

Fig. 1. Detection of p38 mitogen-activated protein kinase (MAPK) and activation in experimental diabetes. Glomeruli were isolated by differential sieving from the renal cortex of control Sprague-Dawley rats (C) or from rats with experimental diabetes induced by an injection of streptozotocin and treated with insulin injected subcutaneously twice daily (total 25IU/kg/day) to maintain normoglycemia (STZ-D + insulin) or untreated (STZ-D) for four weeks. Glomeruli were rapidly lyzed, and an equal quantity of lysate protein was Western immunoblotted (IB) antiphospho(Thr 180, Tyr182) p38MAPK antibody (*A*). Immunoblots were reprobed with a p38MAPK antibody (*B*). Standard phospho-p38MAPK and p38MAPK protein prepared from C6 glioma cells was included to demonstrate specificity of the immunoblotting antibodies. Control glomerular lysates were similarly prepared after an 18-hour incubation at a raised glucose level. After densitometry, p38MAPK activation was quantitated as phospho-p38MAPK/p38MAPK ratio and expressed as a percentage of control for each experimental cohort. The immunoblots shown are from representative animals within a single treatment cohort. Values for p38MAPK activation in each cohort were combined to give a single value for each treatment, and the results are expressed as mean ± SEM for $N = 4$ cohorts. Statistically significant differences over four separate treatment cohorts or in four independent glomerular preparations incubated over 18 hours are shown as $^*P < 0.05$.

noblotting in animals from four separate treatment cohorts. Over these cohorts, the level of p38MAPK was unchanged. A representative immunoblot of p38MAPK present in lysates of glomeruli rapidly isolated from control, insulin-treated diabetic, and diabetic animals is shown in Figure 1. When p38MAPK activation was determined as the ratio of phospho-p38MAPK to p38MAPK and expressed as percentage of control, there was a significant increase in p38MAPK activation (to 242 ± 25% of control, $P < 0.05$). When compared with control, there was no alteration in the activation of p38MAPK in insulin-treated diabetic animals. An activation of p38MAPK could be shown in control glomeruli exposed to raised glucose levels over 18 hours in culture.

Detection of Hsp25 in glomerular extracts

In isolated untreated diabetic glomeruli, there was a fourfold increase (4.31 ± 0.53-fold, $P < 0.01$, $N = 4$ cohorts) in the abundance of Hsp25. Western immunoblotting (Fig. 2) shows the presence of Hsp25 and some further, as yet uncharacterized, immunoreactive proteins that appear to be differentially regulated by the diabetic condition. Over four separate experimental cohorts, Hsp25 was significantly reduced following the *in vivo* treatment of diabetic animals with insulin to restore normoglycemia. In nondiabetic (control) glomeruli, an increase in Hsp25 was seen under conditions of oxidative stress induced by increasing concentrations of H_2O_2 (Fig. 3). This increase occurred over one hour concomitant with an increase in the activation of p38MAPK.

Activation of p38MAPK in treated mesangial cells

Phosphorylation of p38MAPK was investigated in cultured mesangial cells under conditions that would interfere with glutathione status, a major antioxidant defense of the microvascular cell. The addition of BSO, a γ-glutamyl cysteine synthetase inhibitor, increased the sensitivity of p38MAPK activation in the presence of H_2O_2 (Fig. 4). Inclusion of BSO at H_2O_2 concentrations 100 μmol/L and 200 μmol/L, where minimal p38MAPK activation is seen in the presence of H_2O_2 alone, significantly increased p38MAPK activation (2.18- and 2.05-fold, respectively).

Phosphorylation of Hsp25 and size of Hsp25 molecular aggregates in treated mesangial cells

The phosphorylation of Hsp25, immunoprecipitated from cultured mesangial cells, was investigated. In cells metabolically labeled with [^{32}P], Hsp25 phosphorylation was increased in cells treated with H_2O_2 (Fig. 5). For two independent experiments, the relative phosphorylation was calculated as [^{32}P]Hsp25 (arbitrary densitometric units after autoradiography) relative to Hsp25 Western immunoblot (arbitrary densitometric units after ECL),

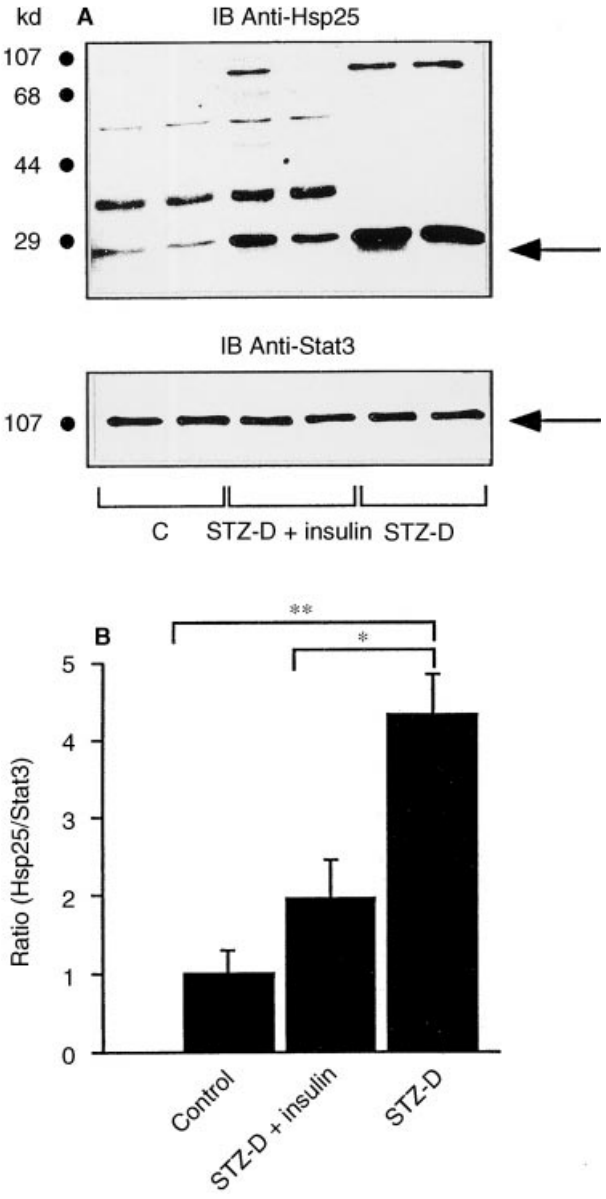

Fig. 2. Detection of heat shock protein 25 (Hsp25) in experimental diabetes. Lysates prepared from glomeruli isolated as described in the **Methods** section and in Figure 1 were Western immunoblotted (IB) with anti-Hsp25 rabbit polyclonal antibody. Immunoblots were reprobed with an anti Stat-3 antibody (shown to be a constitutively activated protein in this glomerular preparation) and after densitometry Hsp25 was quantitated as Hsp25/Stat3 ratio. The immunoblots shown are from representative animals in a single treatment cohort. The statistically significant differences following quantitation of Hsp25/Stat3 ratio over four separate treatment cohorts are expressed as mean ± SEM and are shown as $^{**}P < 0.005$ and $^{*}P < 0.01$.

where the control = 1. The relative phosphorylation of Hsp25 was significantly increased in H_2O_2-treated mesangial cells (5.6-fold). There was no increase in the relative phosphorylation of Hsp25 in cells treated with SB202190, an imidazole derivative cell-permeable inhibitor of p38MAPK activity. In mesangial cells treated concur-

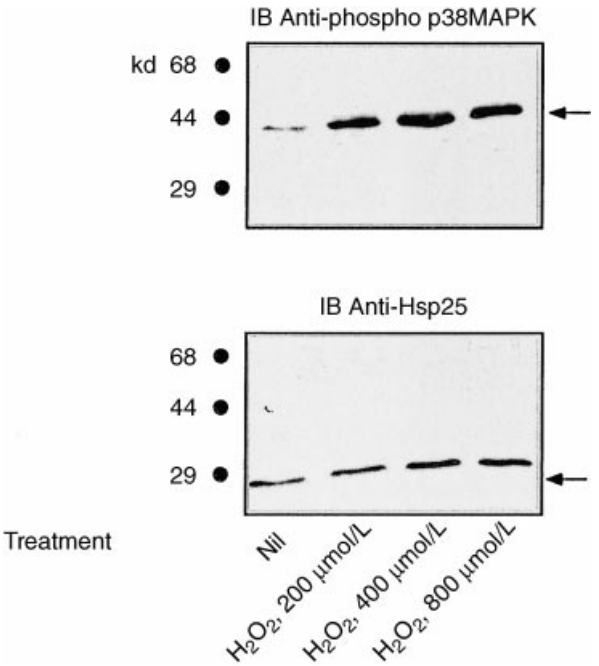

Fig. 3. p38MAPK phosphorylation and detection of Hsp25 in isolated glomeruli following H_2O_2 addition. Glomeruli were treated for one hour with H_2O_2 at the indicated concentrations, lyzed, and immunoblotted (IB) with antiphospho(Thr 180, Tyr182)p38MAPK antibody (upper panel) or anti-Hsp25 antibody (lower panel). This shows a representative immunoblot of two independent experiments with essentially similar findings.

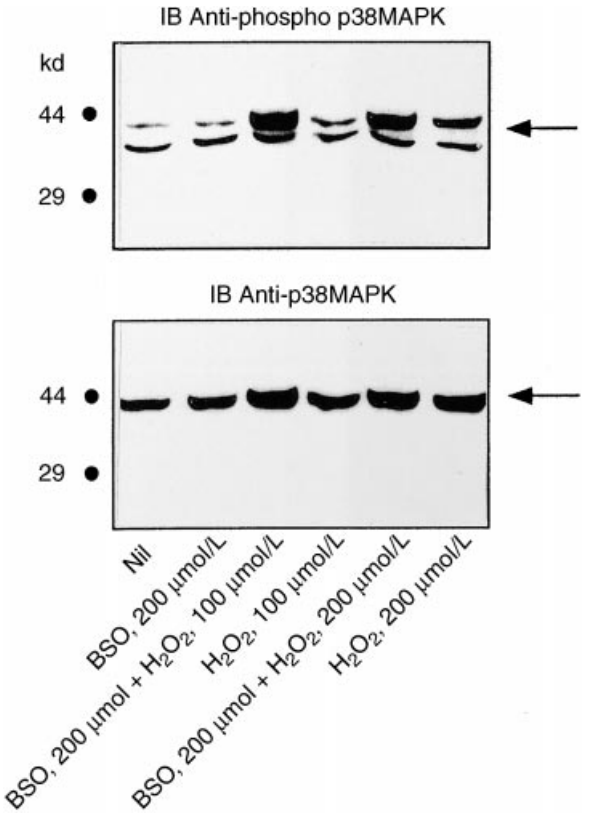

Fig. 4. p38MAPK phosphorylation in response to H_2O_2 following BSO pretreatment of cultured mesangial cells. Mesangial cells were cultured with BSO for 18 hours and exposed to H_2O_2 at the indicated concentrations for 1 hour before lysates were prepared for immunoblotting (IB) with antiphospho(Thr 180, Tyr 182)p38MAPK antibody and p38MAPK antibody. This shows a representative immunoblot of three independent experiments with essentially similar findings.

rently with H_2O_2 and SB202198, relative phosphorylation was reduced compared with H_2O_2 alone (2.6- vs. 5.6-fold, representative data not shown). The addition of BSO alone was without effect on Hsp25 relative phosphorylation. When Sephacryl S300 gel filtration was used to separate Hsp25 in the molecular weight range of >443 to 21 kd, and Hsp25 subsequently immunolocalized by Western blotting after SDS-PAGE under reducing conditions, it was shown that BSO and H_2O_2 reduced the apparent size of Hsp25 to smaller aggregates than seen in untreated cells (Fig. 6A). Under all conditions, the inhibitor of p38MAPK activity sustained large molecular mass aggregates in these cells (SB202190 alone; Fig. 6A).

Phosphorylation of Hsp25 and size of Hsp25 molecular aggregates in glomeruli in experimental diabetes

Glomerular lysates were prepared, and the apparent molecular mass of Hsp25 was determined in glomeruli from nondiabetic (control) and untreated diabetic glomeruli, rapidly isolated to reflect the *in vivo* condition. Apparent molecular mass was consistently shifted toward larger aggregate sizes in control and toward smaller aggregate sizes in untreated-diabetes (representative profiles are shown in Fig. 6B). It should be noted that although this shift in apparent molecular size distribution profile was a consistent finding, the difference in apparent molecular size distribution was less marked in the isolated glomeruli than the shifts seen in mesangial cells treated *in vitro*. The relative phosphorylation of Hsp25 was determined in glomeruli treated for four hours *ex vivo* with [^{32}P]orthophosphate. This labeling time was shown to be sufficient to provide a pool of ^{32}P-ATP for phosphorylation (results not shown). Relative phosphorylation of Hsp25, immunoprecipitated from glomerular lysates, was significantly increased in the untreated diabetic condition when compared with control (1.48 ± 0.180-fold, $N = 5$; representative autoradiograph is shown in Fig. 6C).

Mesangial cell contraction

Differences in the ability of mesangial cells to exert a contractile force within a collagen gel in response to serum stimulation were seen when the planar surface areas of gels were determined (Table 2). Inclusion of

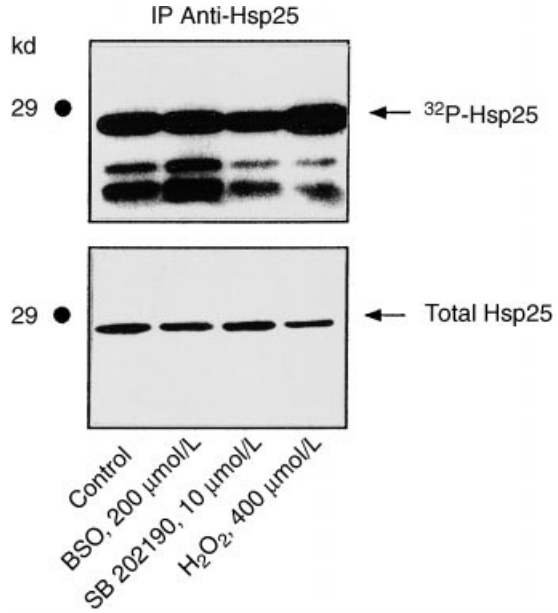

Fig. 5. Hsp25 phosphorylation in cultured glomerular mesangial cells. Cells were metabolically labeled with 0.5 mCi [^{32}P]orthophosphate/10^7 cells for 18 hours with the indicated treatments. Cells were lyzed and immunoprecipitated (IP) with anti-Hsp25 antibody, and the ^{32}P content of Hsp25 was detected by autoradiography after SDS-PAGE and transfer to nitrocellulose (upper panel). Subsequent immunoblotting of this membrane with anti-Hsp25 antibody provided a determination of total Hsp25 in the immunoprecipitate (lower panel). This shows a representative autoradiograph and immunoblot of three independent experiments with essentially similar findings.

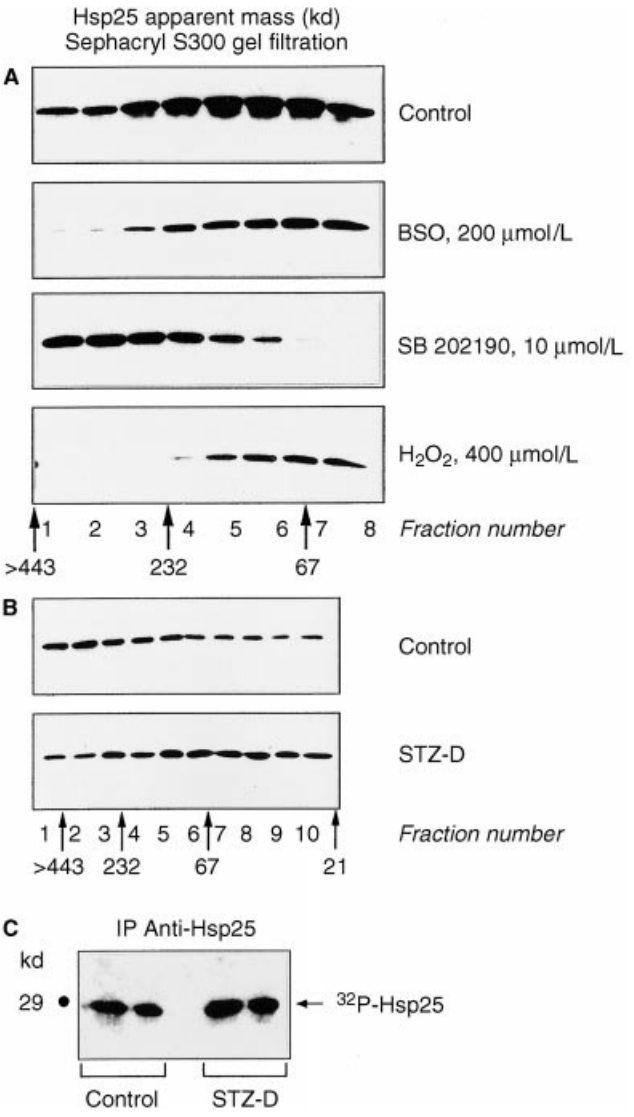

Fig. 6. Sizing of Hsp25 molecular aggregates in treated mesangial cells and in experimental diabetes. Following treatment of cultured mesangial cells with BSO (18 h), SB202190 (18 h), or H_2O_2 (4 h), the Hsp25 aggregates in lysates of cultured mesangial cells were separated by gel filtration with detection of Hsp25 in the indicated column fractions by SDS-PAGE under reducing conditions and immunoblotting (IB) with anti-Hsp25 antibody (*A*). Lysates of glomeruli were rapidly prepared from nondiabetic (control) or untreated diabetic animals (STZ-D) and were similarly isolated (*B*). Arrows indicate the position of molecular mass markers apoferritin (443 kd), catalase (232 kd), bovine serum albumin (67 kd), and soybean trypsin inhibitor (21 kd). This shows representative profiles of three independent experiments. The phosphorylation of ^{32}P-labeled Hsp25 immunoprecipitated from nondiabetic (control) and untreated diabetic glomerular preparations incubated for four hours *ex vivo* with [^{32}P]orthophosphate is shown in an autoradiograph representative of five independent determinations (*C*).

BSO did not have a significant effect on the surface area of these gels, whereas H_2O_2 caused the gel area to be increased significantly, which can be equated in this system with a loss of contraction. This contractile loss was significantly reduced by the inclusion of SB202190. Under these conditions, SB202190 alone had a significant positive effect on contractility.

Contractility of isolated glomeruli

The planar surface area of glomeruli isolated from control and untreated diabetic animals was not significantly different. Additionally, there was no significant difference seen when samples of isolated glomeruli were incubated with SB202190 (control and untreated diabetic) or H_2O_2 (control glomeruli only). Control glomeruli responded to short-term exposure to KCl with a significant reduction in planar surface area, indicating contraction of the glomerulus. No change in planar surface area was seen after KCl in glomerular preparations from untreated diabetic animals, but a significant reduction could be elicited by KCl when these glomeruli were pretreated for four hours with SB20219. In a similar manner, glomeruli from control animals failed to respond to KCl after pretreatment with H_2O_2, but a contractile response to KCl was restored when SB202190 was present over the pretreatment time (Table 3). Glomeruli from untreated diabetic animals were also unresponsive to bradykinin with no change in planar surface area seen (to 10380 ± 154 μm^2 after bradykinin, 100 nmol/L) compared with a significant reduction in planar

Table 2. Contractility of mesangial cell-embedded type I collagen gels

Condition	+ 3% FBS gel area mm^2		
Pre-treatment	Nil	+ BSO 200 μmol/L	+ SB202190 10 μmol/L
Treatment			
Nil	152 ± 10	174 ± 8	122 ± 8[b]
H_2O_2 *100 μmol/L*	168 ± 10	350 ± 10[b]	125 ± 6[b]
H_2O_2 *200 μmol/L*	299 ± 2[a]	364 ± 8[b]	202 ± 4[b]
H_2O_2 *400 μmol/L*	354 ± 9[a]	369 ± 12	235 ± 10[b]

Values for gel planar surface area are expressed as mean ± SEM for 10 independent experiments.
[a] Significant difference from cells without treatment
[b] Significant difference from the equivalent treatment condition without the indicated pretreatment

Table 3. Contractility of isolated glomeruli

	Planar surface area μm^2			
	Control		Untreated diabetic	
Treatment group	Nil	KCl 50 mmol/L	Nil	KCl 50 mmol/L
Pre-treatment				
Nil	9472 ± 140	8146 ± 149[a]	10775 ± 156	10680 ± 181
SB 202190 *10 μmol/L*	9465 ± 198	7815 ± 242[a]	10140 ± 140	9436 ± 201[a]
H_2O_2 *100 μmol/L*	9171 ± 250	9165 ± 298	—	—
H_2O_2 *200 μmol/L*	8965 ± 247	9160 ± 194	—	—
H_2O_2 *200 μmol/L* + SB 202190 *10 μmol/L*	9324 ± 171	8004 ± 172[a]	—	—

Values for glomerular planar surface area are expressed as mean ± SEM (50 to 65 glomeruli at each condition).
[a] Significant difference from equivalent pretreatment without KCl

Table 4. Glomerular protein carbonyl content

	Control	Untreated diabetic
Treatment group	Carbonyl content *nmol/mg protein*	
Pretreatment		
Nil	0.51 ± 0.05	1.24 ± 0.12[a]
H_2O_2 *100 μmol/L*	0.92 ± 0.10[b]	—
H_2O_2 *200 μmol/L*	1.34 ± 0.21[b]	—

Values are shown as mean ± SEM for eight independent determinations at each condition.
[a] Significant difference from control
[b] Significant difference from control without pretreatment

surface area in control glomeruli [to 8054 ± 204 μm^2 after bradykinin (55 glomeruli), $P < 0.005$]. Compared with the planar surface area of control glomeruli without pretreatment (Table 3), an overall reduction in planar surface area of 15% is seen in control glomeruli following short-term exposure to bradykinin.

Protein carbonyl content to determine protein oxidation

Carbonyl groups can be introduced into proteins by oxidative modification. Proteins from glomeruli isolated from untreated diabetic animals contained significantly more DNPH-reactive dicarbonyl groups than glomeruli isolated from control animals (Table 4). Additionally, when control glomeruli were exposed to H_2O_2 over four hours, there was a significant concentration-dependent increase in protein carbonyl content (Table 3).

DISCUSSION

In this study, the finding of an increase in small Hsp25 and an alteration in its oligomeric size and phosphorylation may have significant implications for the diabetic condition. By analogy, with investigations in other systems, these findings may provide an underlying mechanism for the widely described functional deficit in glomerular and mesangial cell contractility in early diabetes [6, 15–18] where an oxidative stress is present. An oxidative stress-induced F-actin reorganization, mediated by the p38MAPK/MAPKAPK-2/small Hsp pathway, is proposed to create a stress-resistant state in a number of cellular models, and to provide a protective situation in which cytotoxicity and apoptosis are not an inevitable outcome of activation of stress-activated kinases [5]. In a renal context, this may be a mechanism providing protection in seemingly diverse conditions of hypertonic stress [28], experimental nephrotic syndrome [29], and renal ischemia [30]. In each of these conditions, there is increased expression of small Hsps and a proposed regulation of pathophysiologic cytoskeletal changes. We propose that, paradoxically, this conferred resistance of the cytoskeleton may explain a well-described early renal diabetic condition of glomerular and mesangial cell hy-

pocontractility, a condition that contributes to a potentially damaging unregulated hyperfiltration in the glomerulus [reviewed in 31]. Actin-containing microfilaments that predominate in the pericapillary extensions of mesangial cells allow the cell to sense any capillary distending force and, by isometric contraction, prevent further glomerular distention [32]. A reduction in the contraction of mesangial cells has been shown in modeled diabetic conditions [6, 15, 33] and in response to reactive oxygen species following puromycin aminonucleoside treatment [25]. The modeled system, which determines mesangial cell contraction within a collagen matrix, requires that contractile forces are transmitted to a matrix of dense collagen fibers via mesangial cell-matrix attachments [24, 25]. A loss of contraction may occur by the direct disruption of actin filaments or by the failure of actin filaments to generate the elastic force to achieve gel contraction. This latter effect may be expected if phosphorylated small Hsp induced stabilized microfilaments. We show that mesangial cell and glomerular hypocontractility seen in the presence of H_2O_2 can be significantly alleviated and contraction restored by addition of SB202190, an inhibitor of p38MAPK activity. Glomeruli from untreated diabetic animals are unresponsive to a pressor agonist (bradykinin) and to KCl. Contractility to KCl, a receptor-independent stimulus, was restored by inhibition of p38MAPK, supporting restoration of a generalized contractile deficit. Investigation of the effect of SB202190 was not extended to study the short-term response of receptor-dependent pressor agonists. It has been shown that pressor agonists such as endothelin 1 [34], angiotensin II [35], and kinins [36] can activate p38MAPK as a rapid response in cardiovascular cells. We would speculate that this has the potential to modify short-term contractile responsiveness by the same mechanism proposed for oxidative stress.

Investigations in murine NIH 3T3 cells transfected with wild-type human Hsp27 and with constructs containing nonphosphorylatable mutants have shown that serine residues 15, 78, and 82 are key to the control of Hsp27 oligomeric structure [8]. A similar change in Hsp oligomer size has been described in murine fibrosarcoma cells transfected with human Hsp27 or NIH 3T3 cells transfected with murine Hsp25 (phosphorylated serines 15 and 86) treated with H_2O_2 or with BSO, where a depletion in glutathione may contribute to an increase in the intracellular levels of H_2O_2 [7, 8] or most recently in H_2O_2-treated cultured neonatal ventricular myocytes [11]. Additionally, a protection against tumor necrosis factor-α (TNF-α)–induced cytotoxicity, seen in Hsp-27-transfected cells, required that Hsp27 be maintained in the large oligomeric state. In this latter condition, there is a reduction in reactive oxygen species and an increase in cellular glutathione content [8]. These authors have proposed that this may be due to the ability of large molecular aggregates of small Hsp to act as a chaperone for enzymes of glutathione synthesis, thus protecting folding intermediates, as is seen for other non-native proteins in heat shock [37], but this is not currently known. The alteration of small Hsp oligomeric size distribution, shown in this study in diabetes, would not favor protection through this mechanism and could underlie a glucose-induced reduction in glutathione in vascular and other cells [38, 39] and a loss of glutathione-dependent protection from over production of reactive oxygen species. Thus, despite an increase in the abundance of Hsp25 in experimental diabetes, a shift in the oligomeric size to smaller phosphorylated forms may compromise any protective response to oxidative stress and may concomitantly contribute to a loss of microfilament reactivity. It has been shown that a stress-induced phosphorylation and disaggregation provides the only condition in which it is possible for small Hsps to modulate the actin microfilament response to reactive oxygen species [40].

Activation of other MAPKs, specifically p42MAPK [or extracellular signal-regulated kinase-2 (ERK-2)], has been shown in glomeruli isolated from STZ-induced diabetic rats and in isolated glomeruli cultured in high-glucose conditions for five days [41]. However, Zent, Ailenberg, and Silverman have shown recently that in the model of serum-induced mesangial cell contraction of a collagen gel, ERK-2 is tyrosine phosphorylated, but gel contraction is independent of this ERK-2 phosphorylation and activation [42].

Protein kinase C is also activated in many tissues, including the glomerulus [3, 43], in diabetes and in mesangial cells in response to raised glucose [44]. There is considerable evidence for an involvement of the PKCβII isoform, based on the efficacy of a specific inhibitor of PKCβII to abolish or reduce a number of microvascular, macrovascular, and cardiac sequelae of diabetes [1, 45]. It is not known whether PKCβII contributes to the phosphorylation of small Hsps in diabetes, but it has been shown that, of recombinant PKC isoforms tested, the δ isoform is an effective Hsp kinase, whereas the βI, βII, ϵ, and ζ isoforms are poor small Hsp25/27 kinases [21]. A possible role for PKCδ in small Hsp phosphorylation should be considered, but may differ between tissues. Myocardial particulate PKCδ is increased in an early cardiomyopathy seen in the BB/W or diabetic rat [46] and by PKC activity raised by increased glucose in rat aortic smooth muscle cells overexpressing PKCδ [47]. However, PKCδ is not altered in the glomerulus in diabetes [45], although increased PKCδ immunofluorescence, distributed in a cytoskeletal pattern, is seen in mesangial cells exposed to raised glucose in the short term in which F-actin assembly/disassembly is found [33]. In this study, beyond the demonstration of p38MAPK activation, the effect of raised glucose levels has not been investigated further. Rather, the direct effects of an oxidative stress

have been considered. It would appear that a reduction in Hsp25 molecular aggregate size, H_2O_2-induced small Hsp phosphorylation, and H_2O_2-induced loss in mesangial cell contractile responsiveness is effected by a p38MAPK-dependent pathway rather than a PKC-dependent pathway, at least as indicated by the p38MAPK inhibitor SB202190. SB202190 is widely regarded as a highly effective inhibitor of p38MAPK, and although some lack of exclusivity among the stress-related kinases should be considered [48], PKC activation is not inhibited by SB202190 [49].

In the glomerulus, a coordinated small Hsp response to treatment with H_2O_2 or experimental diabetes is an apparent increase in Hsp25 abundance together with a p38MAPK-related phosphorylation. A rapid increase in Hsp25 abundance is seen in H_2O_2-treated glomeruli. This increase, occurring within one hour, may be accounted for by an increase in Hsp25 expression, as metabolic labeling of cellular protein with [^{35}S]methionine is reported rapidly after heat stress [50]. However, by analogy with other systems, it may be more likely that subcellular relocalization could contribute to this effect. In cardiac myocytes, Hsp25 is found at cytoplasmic, cytoskeletal, and nuclear locations with redistribution directly after heat shock not only to the cytoskeleton but also to the nucleus [51]. This study employs experimental conditions that determine Hsp25 at both cytosolic and cytoskeletal locations. Treatment with H_2O_2 results in a marked reduction in molecular aggregate size and an increase in phosphorylation, which would favor translocation to the actin cytoskeleton and thus retention in a cytosolic/cytoskeletal fraction. In addition, the antibody used in this study raised to recombinant mouse Hsp25 has been shown recently to cross-react with previously unrecognized mitochondrial small Hsps (~35 kd) in rat adrenal pheochromocytoma cells. An increase in these small Hsps is proposed to protect mitochondrial complex I from oxidative stress [52]. A decrease in a cross-reactive species (~35 kd) in untreated diabetes, seen in all cohorts in this study, remains to be investigated.

The mechanism for the observed increase in small Hsp25 over a protracted period of experimental diabetes is unknown and may involve cytosolic/cytoskeletal retention as conditions favoring this are also shown. However, a longer term response to increase small Hsps may include activation of the transcription factor, heat shock factor-1 (HSF-1), for which activation involving trimerization, phosphorylation, and nuclear translocation has been extensively described in conditions of oxidative stress [53] and can follow the formation of proteins with non-native glutathione-mixed disulfides and destabilization of cellular proteins [54]. Participation of this transcription factor could have more far reaching consequences, as HSF-1 may augment synthesis not only of the small Hsps but of several other Hsps with diverse chaperone functions [55]. There is currently considerable interest in the expression and tissue localization of small Hsps in experimental renal perturbations with direct relevance to human disease [28–30] and in human disease states, including increased localization to tumor microvascular endothelial proliferations and blood vessels [56] and alterations to cardiac tissue that occur in the diseased heart [57]. Although not considered previously, these aspects and their implications should now be considered in the renal glomerulus in diabetes.

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APPENDIX

Abbreviations used in this article are: BSO, buthionine sulfoximine; DMEM, Dulbecco's modified Eagle's medium; DNPH, 2,4-dinitrophenylhydrazine; ERK-2, extracellular signal-regulated kinase-2; FBS, fetal bovine serum; GSH, glutathione; HSF-1, heat shock factor-1; Hsp, heat shock protein; MAPKAPK-2, mitogen-activated protein kinase-activating protein kinase-2; PKC; protein kinase C; PVDF, polyvinylidene difluoride; p38MAPK, p38 mitogen-activated protein kinase; SB202190, 4-(4-fluophenyl)-2-(4-hydroxyphenyl)-5-5(4-pyridyl)1H-imidazole; STZ, streptozotocin.

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